RESEARCH ARTICLE

Bacterial diversity on the surface of potato tubers in soil and the influence of the plant genotype

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Abstract

The surface of tubers might be a reservoir for bacteria that are disseminated with seed potatoes or that affect postharvest damage. The numbers of culturable bacteria and their antagonistic potential, as well as bacterial community fingerprints were analysed from tubers of seven field-grown potato genotypes, including two lines with tuber-accumulated zeaxanthin. The plant genotype significantly affected the number of culturable bacteria only at one field site. Zeaxanthin had no effect on the bacterial plate counts. In dual culture, 72 of 700 bacterial isolates inhibited at least one of the potato pathogens *Rhizoctonia solani*, *Verticillium dahliae* or *Phytophthora infestans*, 12 of them suppressing all three. Most of these antagonists were identified as *Bacillus* or *Streptomyces*. From tubers of two plant genotypes, including one zeaxanthin line, higher numbers of antagonists were isolated. Most antagonists showed glucanase, cellulase and protease activity, which could represent mechanisms for pathogen suppression. PCR-DGGE fingerprints of the 16S rRNA genes of bacterial communities from the tuber surfaces revealed that the potato genotype significantly affected the *Pseudomonas* community structure at one site. However, the genotypes showed nearly identical fingerprints for *Bacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Bacillus* and *Streptomycetaceae*. In conclusion, tuber-associated bacteria were only weakly affected by the plant genotype.

Introduction

The potato tuber represents a belowground part of the shoot that clearly differs from the roots anatomically (Fernie & Willmitzer, 2001). While microorganisms colonizing the rhizosphere have access to exudates such as sugars, amino acids and organic acids, which are excreted by the plant (Sørensen, 1997; Bais *et al.*, 2006), nutrient availability on the tuber surface is supposed to be mainly related to cells decaying during tuber growth or lesions (Lottmann *et al.*, 2000). Tubers represent a primary infection source for many pathogens of potato plants that at least temporarily colonize the tuber surface (van der Wolf & De Boer, 2007). Because infected tubers often remain symptomless, pathogens are dispersed over long distances by seed tubers. During the growth of the tuber, its surface might be colonized by a complex microbial community, parts of which have the potential to antagonize phytopathogens (Clulow *et al.*, 1994). However, this has hardly been studied so far. Most previous studies on microorganisms associated with the potato tuber focused on endophytic bacteria (Sturz *et al.*, 1999) or on bacterial and fungal pathogens of potato tuber diseases such as *Streptomyces scabies* (Loria *et al.*, 1997) or *Dickeya* species (Dowley & O'Sullivan, 1991; Pérombelon, 2002; Gudmestad *et al.*, 2007). Only Lottmann *et al.* (1999) studied bacterial isolates from the tuber surface. They characterized 42 bacteria that *in vitro* antagonized *Verticillium dahliae* or *Erwinia carotovora*. Despite the obvious potential to control tuber-disseminated potato diseases through antagonistic communities, serious efforts have not yet been made to study the impact of the potato genotype and other factors on the tuber-associated bacterial communities.

The potato genotype was shown to be one of the factors shaping the associated microbial community in the rhizosphere (Becker *et al.*, 2008; Weinert *et al.*, 2009; Andreote *et al.*, 2010). Even minor genotype differences as between genetically modified (GM) and parental potato lines are believed to affect the microbial colonization of the plants (Heuer *et al.*, 2002; Rasche *et al.*, 2006; van Overbeek & van Elsas, 2008; Weinert *et al.*, 2009). Potato plants accumulating the carotenoid zeaxanthin in their tubers were designed to serve as a functional food counteracting age-related macular degeneration (Snodderly, 1995; Römer *et al.*, 2002). Potato plants normally transform zeaxanthin to violaxanthin, which acts as a UV protectant. The fully developed tubers of the GM plants contain high amounts of zeaxanthin (Römer *et al.*, 2002). It is not well understood whether high concentrations of such compounds in the tuber could affect associated bacterial communities.

The objective of this study was to investigate the influence of the plant genotype on the bacterial community composition on the tuber surface, especially with respect to bacterial antagonists of potato pathogens. Five potato cultivars and two potato lines, which accumulate zeaxanthin in their tubers, were investigated in field experiments at two sites. The numbers of culturable bacteria, their antagonistic potential towards *Rhizoctonia solani*, *V. dahliae* and *Phytophthora infestans* and the putative mechanisms of this antagonism were analysed. In a cultivation-independent approach, the composition of various bacterial taxonomic groups on the tuber surface of the potato genotypes was compared by statistical analysis of PCR-denaturing gradient gel electrophoresis (DGGE) fingerprints.

Materials and methods

Field experiments and sampling

Five commercial potato cultivars (*Solanum tuberosum* L.), 'Baltica', 'Selma', 'Désirée', 'Ditta' and 'Sibu', and two GM lines were grown in a randomized block design in Southern Germany at the Oberviehhausen site (12.75/12°45′0″E; 48.7333/48°44′0″N) in 2006 and at the Roggenstein site (11.25/11°15′0″E; 48.1833/48°11′N) in 2007. The two GM lines were modified from their parental cultivar 'Baltica' by cosuppression (line SR47) or antisense (line SR48) to accumulate the carotenoid zeaxanthin in their tubers, but not in leaves or stems (for details, see Römer *et al.*, 2002). The modified plants contained zeaxanthin levels in the tuber of up to 40 $\mu g\,g^{-1}$ dry weight (dw) (SR47) and 17 $\mu g\,g^{-1}$ dw (SR48) in comparison with 0.2 $\mu g\,g^{-1}$ dw of the cultivar 'Baltica'. The soil characteristics of both sites and the experimental field design were described recently by Weinert *et al.* (2009). In brief, although the pH of both soils was rather similar, other soil parameters differed considerably. Thus, the Roggenstein soil was composed of 26.1% sand, 44.0% silt, 28.1% clay, 1.1% C_{org} and 0.1% N_t, while the Oberviehhausen soil was composed of 54.6% sand, 31.3% silt, 14% clay and contained 1.9% C_{org} and 0.2% N_t. The plants were treated several times during the season with fungicides against *P. infestans*, and appeared to be healthy at the time of sampling.

The fully developed tubers were sampled at the senescent stage of the plants (stage EC90, according to Hack *et al.*, 1993). For each of four replicate field plots per plant genotype, five tubers of equal size (one tuber per sampled plant) with tightly adhering soil were collected to form one composite sample per plot. In total, 56 samples were analysed (two field sites, seven plant genotypes and four replicate plots). After the removal of coarse soil particles, the outer peel layer (ectodermis) with tightly adhering soil was carefully stripped off with a sterile spatula. Hence, the tuber surface comprised microbial communities colonizing the soil attached to the tuber surface, but also those colonizing the potato tuber ectodermis. Five grams of peel material was transferred into sterile Stomacher bags and homogenized with 15 mL Milli-Q water for 60 s in a Stomacher laboratory blender (Seward, West Sussex, UK) at high speed. The homogenization step was repeated three times and the combined suspensions were collected in 50-mL tubes. The suspensions containing bacteria from the outer peel layer were centrifuged at 4 °C for 10 min and 800 ***g*** to sediment coarse soil particles. One millilitre of the supernatant was taken for culturing bacteria on agar plates, and the rest of the microbial suspension was pelleted (15 min, 10 000 ***g***, 4 °C) to extract total DNA for molecular analysis. Serial dilutions were spread onto R2A agar plates (Merck, Darmstadt, Germany) supplemented with 100 $\mu g\,mL^{-1}$ cycloheximide to prevent fungal growth. The CFUs were counted after 5 days of incubation at 28 °C. Log-transformed bacterial CFU counts were tested for significant effects of the plant genotypes using ANOVA for the global comparison, and the Tukey test for pairwise comparisons.

Dual-culture assays to screen for *in vitro* antagonistic strains

To investigate the antagonistic potential of the cultured bacteria, 25 colonies per replicate plot from one field site (Oberviehhausen) were picked and repeatedly streaked for purification, resulting in 100 isolates per plant genotype and a total of 700 strains. A stock was archived at −70 °C. All 700 bacterial isolates were tested in dual-culture assays for their *in vitro* antagonistic potential towards three major potato pathogens: *R. solani* Kühn AG-3 (*Basidiomycetes*), *V. dahliae* Kleb. ELV25 (*Ascomycetes*) and *P. infestans* (Mont.) De Bary (isolate 20/01) (*Oomycetes*). The three pathogens were obtained from the strain collections of Graz University of Technology and

Julius Kühn-Institut, Braunschweig. Dual-culture assays were performed as described recently (Weinert *et al.*, 2010). Briefly, disks of *R. solani* grown on Waksman agar (WA) or *P. infestans* grown on pea agar were placed in the corners of a fresh WA or pea agar plate, respectively, and four bacterial isolates were streaked between the disks. *Verticillium dahliae* hyphal suspension was plated on WA plates and four different bacterial strains were streaked on the same plate. The results of all dual-culture assays were confirmed by a second independent assay. The numbers of isolated antagonists were tested for significant differences between plant genotypes using the χ^2 test of the procedure PROC FREQ of the statistical software SAS 9.1 (SAS Institute, Cary, NC).

Genetic characterization and identification of bacterial antagonists

After enzymatic and chemical lysis according to the manual of the genomic DNA extraction kit from Qiagen (Hilden, Germany), the DNA of *in vitro* antagonistic strains was extracted using the UltraClean™ 15 DNA Purification Kit (Mo Bio Laboratories, Carlsbad, CA) as described previously (Heuer & Smalla, 2007). In order to select representative strains for 16S rRNA gene sequence-based identification, *in vitro* antagonists were compared by amplified ribosomal DNA restriction analysis (ARDRA), as well as by BOX-PCR fingerprinting, which sensitively detects genome differences between strains of a species. For ARDRA, the 16S rRNA gene was amplified from genomic DNA with the primers F27 and R1492 (Weisburg *et al.*, 1991), followed by a double digest of the amplicons with 5 U of the enzymes Hin6I and Bsh1236I (Fermentas) for 3 h at 37 °C. Fifteen microlitres of the restriction digest was separated in a 4% agarose gel (NuSieve 3 : 1) in 0.5 × TBE buffer for 4 h, stained with ethidium bromide and visualized using a UV-transilluminator. To identify clonal strains putatively, BOX-PCR was carried out for all *in vitro* antagonistic isolates displaying similar ARDRA patterns. BOX-PCR was performed using the BOX_A1R primer as described previously by Rademaker *et al.* (1999). Eight microlitres of the PCR products were run on 1.5% agarose gels in 0.5 × TBE buffer for 4 h, stained with ethidium bromide and photographed under UV. Cluster analysis of ARDRA and BOX fingerprints was performed by the unweighted pair group using the average linkages method based on the Dice correlation using the GELCOMPAR II program version 4.5. (Applied Maths, Kortrijk, Belgium). Antagonistic isolates were assumed to represent the same type when ARDRA and BOX patterns showed at least 85% similarity. *In vitro* antagonists with unique ARDRA or BOX patterns were identified by comparison of approximately 700 bp of the 16S rRNA gene with sequences deposited in GenBank using the basic local alignment and search tool BLASTN.

Determination of enzymatic properties, siderophore- and *N*-acyl homoserine-lactone (AHL)-production

Some characteristics were analysed in depth to provide further insights into the traits potentially responsible for the antagonistic activity of the bacterial isolates. β-Glucanase and cellulase activities were determined using chromogenic AZCL and reamazol brilliant blue R (AZO) substrates, respectively (Megazyme International, Ireland). Blue and white haloes were measured after incubation for 5 days at 28 °C. Protease activity (casein degradation) was determined from clearing zones in skim milk agar (400 mL sterilized skim milk mixed with 1/10 trypticase soy broth and 16 g Bacto agar at 55 °C) after 5 days of incubation at 28 °C. Chitinolytic activity was tested in chitin minimal medium as described by Berg *et al.* (2001). Clearance haloes indicating β-1,4-glucosamine polymer degradation were measured after 7 days at room temperature. The production of siderophores under Fe^{3+}-limited conditions was analysed using the plate assay developed by Schwyn & Neilands (1987). The size of orange haloes formed around the streak was measured after 3 days of incubation at room temperature. The production of AHLs was investigated in a cross-streak assay using the bioluminescent sensor plasmid pSB403 in *Escherichia coli* to detect long-chain AHL (3-oxo-C6) molecules produced by the bacterial *in vitro* antagonists (Winson *et al.*, 1998). In a second test, the sensor strain *Chromobacterium violaceum* CV026 was used (McClean *et al.*, 1997) that changes its colour to purple in the presence of short-chain AHLs. *Serratia plymuthica* HROC48 (Liu *et al.*, 2007; Müller *et al.*, 2009) was used as a positive control in both assays.

Extraction of total community (TC) DNA from tuber surface samples

TC DNA was extracted from 0.5 g of the pellets obtained from the tuber surface of potato plants grown at both sites. After initial cell lysis using the FastPrep FP120 bead beating system (QBiogene, Carlsbad, CA) for 30 s at a high speed (this step was repeated twice), the DNA was extracted by means of the BIO-101 DNA spin kit for soil (QBiogene) according to the instructions of the manufacturer. The extracted DNA was further purified using the Geneclean Spin Kit (QBiogene). Yields of genomic DNA were checked on a 0.8% agarose gel and photographed under UV after staining in ethidium bromide. After estimating the DNA yield using the 1-kb plus DNA ladder (Invitrogen, Karlsruhe, Germany), the DNA was diluted 1 : 5 with elution buffer of the purification kit.

PCR amplification of bacterial 16S rRNA and *Pseudomonas*-specific *gacA* genes and DGGE analysis

TC DNA was used as a template for the amplification of the bacterial 16S rRNA gene using the primer pair F984GC/R1378 as described by Heuer *et al.* (1997). All primer sequences used for the specific amplification of different bacterial groups and the *Pseudomonas gacA* gene fragment in a nested-PCR approach are given in Table 1. The products of the first PCR were diluted (1 : 10 or 1 : 20) and used as templates for the F984GC/R1378 PCR with 20 (*Streptomycetaceae*, *Bacillus*) or 25 (*Actinobacteria*, *Alpha-* and *Betaproteobacteria*, *Pseudomonas*, *gacA* gene) cycles. To identify bands in the community DGGE profiles that have an identical migration as PCR products of the major antagonistic species (*Bacillus pumilus*, *Bacillus subtilis*, *Streptomyces bobili* and *Streptomyces flavolimosus*), genomic DNA extracted from these antagonists served as a template for amplification with the primer pair F984GC/R1378. DGGE was performed with a PhorU$_2$ apparatus (Ingeny, Goes, the Netherlands) using a double gradient for both 16S rRNA and *gacA* gene separation as described previously by Weinert *et al.* (2009).

Statistical analysis of DGGE community fingerprints

DGGE community fingerprints with four independent replicates of each of the seven plant genotypes loaded in random order were analysed using the GELCOMPAR II program version 4.5 as described previously (Smalla *et al.*, 2001). In brief, after normalization and background subtraction, the Pearson correlation index for each pair of lanes within a gel was calculated as a measure of similarity between the community fingerprints. The resulting similarity matrix was used to test for significant treatment effects ($P < 0.05$) using a permutation test as described by Kropf *et al.* (2004). The difference in fingerprints between treatment groups was calculated as (1 plus average pairwise Pearson's correlation within the groups minus average pairwise Pearson's correlation between the groups). This method avoids band assignment and quantification and is therefore more reliable and less subjective for analysing DGGE fingerprints.

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study were deposited in the GenBank database under accession numbers FJ845010–FJ845052.

Results

Bacterial counts on the tuber surface

Bacterial CFU counts determined for samples obtained from the tuber surface of seven plant genotypes grown at the Oberviehhausen site in 2006 ranged from 1×10^7 g^{-1} tuber peel fresh weight for the cultivar 'Ditta' to 3×10^7 g^{-1} for 'Baltica' and the two GM lines (Table 2). While the differences in CFUs between plant genotypes were not significant at this site ($P = 0.07$, $n = 4$, ANOVA), a significant effect of the plant genotype could be shown at the Roggenstein site ($P = 0.005$). Here, the CFUs from the cultivar 'Selma' were significantly lower compared with the cultivars 'Ditta', 'Baltica' and both GM lines (Tukey's test, Table 2). On an

Table 1. Primers used in this study

Primer	Sequence 5′–3′	Specificity	Reference
F984	AACGCGAAGAACCTTAC	*Bacteria*	Heuer *et al.* (1997)
GC-clamp	CGCCCGGGGCGCGCCCCGGGCGGGGCG GGGGCACGGGGGG	–	Nübel *et al.* (1996)
R1378	CGGTGTGTACAAGGCCCGGGAACG	*Bacteria*	Heuer *et al.* (1997)
F243	GGATGAGCCCGCGGCCTA	*Actinobacteria*	Heuer *et al.* (1997)
F203α	CCGCATACGCCCTACGGGGGAAAGATTTAT	*Alphaproteobacteria*	Heuer & Smalla (1999)
F948β	CGCACAAGCGGTGGATGA	*Betaproteobacteria*	Gomes *et al.* (2001)
F27	AGAGTTTGATCMTGGCTCAG	*Bacteria*	Weisburg *et al.* (1991)
R1492	TACGGYTACCTTGTTACGACTT	*Bacteria*	Heuer *et al.* (1997)
F311Ps	CTGGTCTGAGAGGATGATCAGT	*Pseudomonas*	Milling *et al.* (2004)
R1459Ps	AATCACTCCGTGGTAACCGT	*Pseudomonas*	Milling *et al.* (2004)
BacF	GGGAAACCGGGGCTAATACCGGAT	*Bacillus*	Garbeva *et al.* (2003)
F126	GCCCTGCACTCTGGGACAAGC	*Streptomycetaceae*	Weinert *et al.* (2009)
R1423	GTTAGGCCACCGGCTTCG	*Streptomycetaceae*	Weinert *et al.* (2009)
gacA-1F	TGATTAGGGTGYTAGTDGTCGA	*gacA* gene	Costa *et al.* (2007)
gacA2	MGYCARYTCVACRTCRCTGSTGAT	*gacA* gene	de Souza *et al.* (2003)
gacA-1FGC	GC-clamp+GATTAGGGTGCTAGTGGTCGA	*gacA* gene	Costa *et al.* (2007)
gacA-2R	GGTTTTCGGTGACAGGCA	*gacA* gene	Costa *et al.* (2007)

average, the bacterial counts at the Roggenstein site were nearly one order of magnitude lower compared with those at Oberviehhausen.

Identification and characterization of bacteria with antagonistic potential

To investigate the antagonistic potential of the cultured bacteria, 100 isolates per plant genotype from the field site in Oberviehhausen were characterized in dual-culture tests with *R. solani* AG-3, *V. dahliae* V25 and *P. infestans* 20/01. A total of 72 strains of the 700 isolates tested displayed *in vitro* antagonistic activity towards at least one of the three phytopathogens (Table 3). The highest number of strains (54) was active against *P. infestans*. Twelve isolates showed antagonistic activity against all three pathogens. The χ^2 test revealed a significant effect of the plant genotype on numbers of antagonists ($P < 0.001$). The highest numbers of antagonists were detected for the GM line SR48 and the cultivar 'Ditta' (Table 3).

Molecular characterization of the antagonists revealed 28 ARDRA patterns. Some of the ARDRA groups were further differentiated by BOX fingerprints, so that in total we obtained 44 groups. Partial sequencing of the 16S rRNA gene of isolates with unique ARDRA or BOX patterns and of strains representative for isolates sharing the same BOX patterns revealed that most isolates were affiliated to the genera *Bacillus* and *Streptomyces* (Table 4). The 33 *Bacillus* isolates belonged to three species, with the majority assigned to *B. pumilus* (27). BOX fingerprints generated from genomic DNA of the *B. pumilus* isolates revealed six different BOX types (similarity cut-off of 80%), with 15 isolates belonging to one tight major cluster. Of this cluster, 14 isolates shared a common BOX pattern with *B. pumilus* antagonists isolated from the rhizosphere of the same plants. Antagonists from both microhabitats sharing the same BOX profiles either came from different cultivars or from different replicates of a cultivar in the field, suggesting that *B. pumilus* was very abundant in the soil of the Oberviehhausen field site.

The diversity of the 14 *Streptomyces* isolates was very high as their 16S rRNA gene sequences were affiliated to 10 different species. The 12 isolates displaying *in vitro* antagonistic potential against all three pathogens were phylogenetically related to *Pseudomonas fluorescens* (1), *Lysobacter gummosus* (4), *Ensifer adhaerens* (2) and *Dickeya chrysanthemi* (5). While both *E. adhaerens* isolates originated from SR48 tubers, *L. gummosus* and *D. chrysanthemi* were isolated from the tuber surface of different potato genotypes.

Traits potentially responsible for their antagonistic activity were frequently distributed among the isolates. Cellulose and glucan comprise the major components of the *Phytophthora* cell wall. The majority of antagonists (69/72) inhibited *P. infestans*. Of these, 58 produced extracellular β-1,3-glucanase, 46 cellulase and 42 both enzymes. The cell walls of the fungi *Rhizoctonia* and *Verticillium* are mainly composed of chitin and glucan. Eighteen strains could antagonize *R. solani*, 14 of which also inhibited *V. dahliae*. Of these antagonists, 14 showed glucanase and only five showed chitinase activity. Extracellular protease activity was very common among all the strains (60/72). Siderophore production was less frequently detected (15/72), but was a typical feature of the 12 strains that could suppress all three

Table 2. Log_{10} of bacterial CFU counts g^{-1} tuber peel fresh weight determined for the seven potato genotypes grown at the field sites in Oberviehhausen (2006) and Roggenstein (2007)

Plant genotype	Oberviehhausen log_{10} (CFU g^{-1})	Roggenstein log_{10} (CFU g^{-1})
'Baltica'	7.5 ± 0.1 (a)	6.7 ± 0.1 (a)
SR47	7.5 ± 0.1 (a)	6.8 ± 0.2 (a)
SR48	7.5 ± 0.1 (a)	6.9 ± 0.2 (a)
'Selma'	7.4 ± 0.4 (a)	6.1 ± 0.4 (b)
'Désirée'	7.2 ± 0.2 (a)	6.4 ± 0.3 (ab)
'Ditta'	7.1 ± 0.2 (a)	6.7 ± 0.3 (a)
'Sibu'	7.3 ± 0.1 (a)	6.6 ± 0.2 (ab)

Numbers followed by different letters are significantly different as determined by Tukey.

Table 3. Numbers of obtained antagonists from the sampling site in Oberviehhausen towards the three potato pathogens *Rhizoctonia solani* AG-3 (*R.s.*), *Verticillium dahliae* V25 (*V.d.*) and *Phytophthora infestans* (Mont.) De Bary (20/01) (*P.i.*) as determined using dual-culture *in vitro* assays

Plant genotype	Total number of isolates	Total number of antagonists	Numbers of antagonists towards						
			R.s.	*V.d.*	*P.i.*	*R.s.*+*V.d*	*R.s.*+*P.i.*	*V.d*+*P.i.*	*R.s*+*V.d*+*P.i.*
'Baltica'	100	7	0	0	7	0	0	0	0
SR47	100	4	0	0	3	0	0	0	1
SR48	100	16	0	0	9	0	2	0	5
'Selma'	100	9	0	0	8	0	0	0	1
'Désirée'	100	8	1	0	6	0	0	0	1
'Ditta'	100	24	0	0	18	2	1	0	3
'Sibu'	100	4	0	0	3	0	0	0	1
Total no.	700	72	1	0	54	2	3	0	12

phytopathogens. Especially, all *P. fluorescens* (2) and *D. chrysanthemi* (5) isolates were strong siderophore producers (halo sizes > 1 cm). The ability to produce AHL molecules might indirectly contribute to the antagonistic activity, if genes involved in antagonism are under the control of AHLs. The formation of long-chain AHLs was detected for 10 strains, including all *D. chrysanthemi* (five), the two *E. adhaerens* and three *Streptomyces* isolates. Interestingly, all *D. chrysanthemi* (5) strains could also produce short-chain AHLs.

Table 4. Phylogenetic affiliation of *in vitro* antagonists determined by partial 16S rRNA gene sequencing

Phylum	Number of isolates
Firmicutes	*n* = 33 (48.5%)
Bacillus pumilus	27
Bacillus subtilis	5
Bacillus licheniformis	1
Gammaproteobacteria	*n* = 15 (22.1%)
Pseudomonas fluorescens	2
Lysobacter enzymogenes	3
Lysobacter gummosus	5
Dickeya chrysanthemi	5
High G+C Gram-positive bacteria	*n* = 14 (20.6%)
Streptomyces acidiscabies	1
Streptomyces antibioticus	1
Streptomyces bobili	2
Streptomyces chartreusis	1
Streptomyces diastaticus ssp. *ardesiacus*	1
Streptomyces flaveolus	1
Streptomyces flavolimosus	3
Streptomyces griseoaurantiacus	1
Streptomyces puniceus	1
Streptomyces tubercidicus	1
Tsukamurella pulmonis	1
CFB group bacteria	*n* = 3 (4.4%)
Flavobacterium sp.	1
Flavobacterium pectinovorum	1
Flavobacterium johnsoniae	1
Alphaproteobacteria	*n* = 2 (2.9%)
Ensifer adhaerens	2
Betaproteobacteria	*n* = 1 (1.5%)
Zoogloea ramigera	1
Total no. of identified isolates	68

Impact of the plant genotype on tuber-associated bacterial communities

In a cultivation-independent approach, the composition of various bacterial taxonomic groups on the tuber surface of the potato genotypes was compared. The similarity matrices of the fingerprints obtained by GELCOMPAR were used for the permutation test for significant differences either between the parental cultivar 'Baltica' and the two GM lines, or among all seven plant genotypes. The analysis of samples of the Oberviehhausen site focused on *Bacteria* and on the bacterial groups that comprised the most frequently identified *in vitro* antagonists. Therefore, in addition to the *Bacteria* fingerprints, *Bacillus*, *Streptomycetaceae* and *Pseudomonas* fingerprints were generated. Statistical analysis revealed no significant differences for *Bacteria* as well as the three group-specific fingerprints generated with samples of the Oberviehhausen site (Table 5). Analysis of the samples from the Roggenstein site included a wider range of bacterial groups. Nearly identical fingerprints were found for *Bacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Bacillus*, *Streptomycetaceae* and *gacA* of the genus *Pseudomonas*, which all showed average differences of 1.6% or less. The *Pseudomonas* fingerprints differed on average by 3.4% between all seven plant genotypes (Table 5). Differences among the plant genotypes were highly significant for this group ($P < 0.001$). Pairwise comparisons revealed that 'Selma' and 'Ditta' differed from both 'Baltica' and SR47,

Table 5. Average dissimilarity of bacterial community fingerprints from 'Baltica' and GM lines, or from all potato genotypes, and significance of differences (see Materials and methods)

	Oberviehhausen (2006)		Roggenstein (2007)	
	Comparison of		Comparison of	
DGGE gel	Baltica-GM lines	All genotypes	Baltica-GM lines	All genotypes
Bacteria	0.0	0.3	0.0	1.6
Pseudomonas	0.0	0.0	1.2	3.4*
Actinobacteria	ND	ND	0.0	0.0
Alphaproteobacteria	ND	ND	0.4	0.6
Betaproteobacteria	ND	ND	0.0	0.7
Bacillus	0.0	0.0	0.0	0.0
Streptomycetaceae	0.0	0.0	0.0	0.0
*gac*A	ND	ND	0.0	0.6

*Significant difference ($P \leq 0.05$) as determined using the permutation test.
ND, not determined.

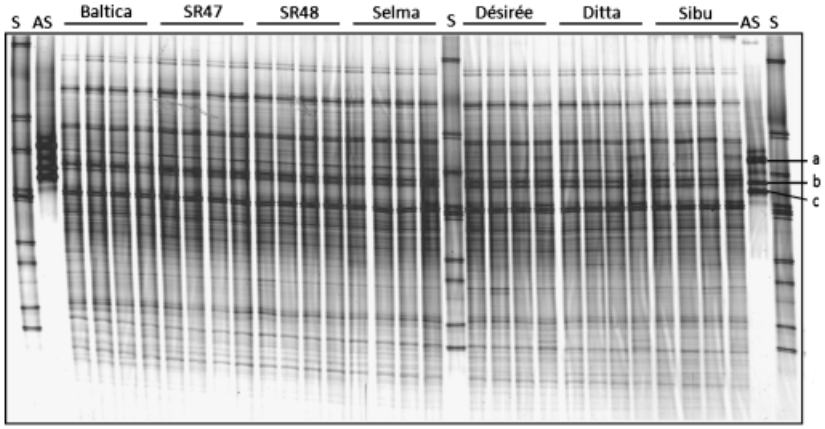

Fig. 1. *Bacillus*-specific DGGE fingerprint of tuber surface samples generated with four independent repetitions of the commercial cultivars 'Baltica', 'Selma', 'Désirée', 'Ditta' and 'Sibu' and the two GM lines SR47 and SR48 grown at the field trial in Oberviehhausen. S, bacterial standard; AS, standard generated with *in vitro* antagonistic strains obtained from the tuber surface. [(a and b) *Bacillus pumilus*, (c) *Bacillus subtilis*)].

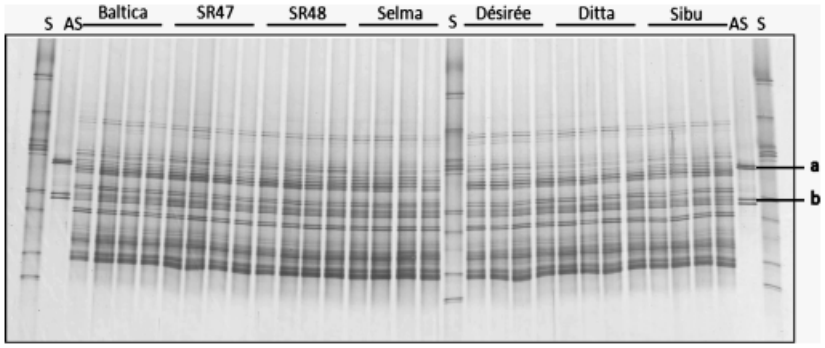

Fig. 2. *Streptomycetaceae*-specific DGGE fingerprint of tuber surface samples generated with four independent repetitions of the commercial cultivars 'Baltica', 'Selma', 'Désirée', 'Ditta' and 'Sibu' and the two GM lines SR47 and SR48 grown at the field trial in Oberviehhausen. S, bacterial standard; AS, standard generated with *in vitro* antagonistic strains obtained from the tuber surface. [(a) *Streptomyces bobili*, (b) *Streptomyces flavolimosus*].

while 'Sibu' was different from all other plant genotypes, except for 'Baltica'. Overall, the differences in DGGE fingerprints between bacterial communities from the various plant genotypes were more pronounced for Roggenstein than for Oberviehhausen. The composition of tuber-associated bacterial communities differed considerably in both field experiments (data not shown). Bacterial fingerprints of the same plant genotype differed on average as much as 27% based on the Pearson correlation between gel lanes. Even stronger differences were observed for tuber-associated *Streptomycetaceae* communities of both field experiments, which exceeded 32%. These differences were highly significant ($P < 0.001$).

We were interested in identifying putative antagonists in the DGGE community patterns. All Gram-positive antagonists that were isolated more than once were checked for their electrophoretic mobility in DGGE. Three strains of the *Firmicutes*, *B. pumilus* 42 and 112 and *B. subtilis* 591, as well as two strains of the *Actinobacteria*, *S. bobili* 149 and *S. flavolimosus* 474, represented the different electrophoretic mobilities of these groups. The 16S rRNA gene PCR products of these strains were loaded next to the respective community fingerprints to identify bands belonging to putative antagonists.

The DGGE band of *B. pumilus* strain 42 comigrated to a prominent band in all of the profiles at a similar intensity, independent of the plant genotype (Fig. 1, band designated b). A band comigrating to *B. subtilis* 591 could be found in the fingerprint of 'Ditta' plot 4. From this plot, three *B. subtilis* antagonists were isolated. The upper DGGE band of the *S. bobili* strain 149 and the two bands of *S. flavolimosus* 474 shared the same electrophoretic mobility as bands appearing in all of the *Streptomycetaceae* profiles, indicating that *S. bobili* and *S. flavolimosus* might belong to the dominant *Streptomycetaceae* populations (Fig. 2).

Discussion

Breeding or genetic modification of potato varieties might be an option to influence the balance of pathogens and antagonists on the tuber surface. In this study, we elucidated the potential of the plant genotype to affect the bacterial colonization of the tuber ectodermis and the tightly adhering soil. The culturable fraction revealed plant genotype effects with regard to the total number of CFUs and the relative numbers of bacteria with antagonistic potential. The majority of antagonists retrieved in this study were affiliated to the Gram-positive genera *Bacillus* and *Streptomyces*, while

all *Verticillium* antagonists were Gram-negative bacteria. Also, in the study of Lottmann *et al.* (1999), > 70% of the *Verticillium* antagonists isolated from the tuber surface were Gram negative and mainly assigned to fluorescent *Pseudomonas* species. Recently, bacterial isolates with antagonistic potential obtained from the rhizosphere of the same potato genotypes were characterized (Weinert *et al.*, 2010). Although similar genera were detected in the rhizosphere, the proportion of *Bacillus* strains was higher for the tuber surface with 48.5%, compared with 12.1% in the rhizosphere (Weinert *et al.*, 2010). In contrast, *Streptomyces* isolates with *in vitro* antagonistic activity were more frequently retrieved from the rhizosphere (51.2% vs. 19.1%). Several antagonists were isolated from both microenvironments, for example *B. pumilus*, *P. fluorescens*, *L. gummosus*, *D. chrysanthemi*, *S. bobili* or *S. flavolimosus*. However, the tuber surface also harboured a number of species with antagonistic properties that were found exclusively in this microenvironment. Differences in the bacterial community structure between rhizosphere and tuber surface can be expected because bacteria in the rhizosphere have access to nutrients that are excreted by the plant while nutrient availability at the tuber surface might be mainly due to lesions. Some of the antagonists obtained from the tuber surface in this study were reported previously as potential biocontrol agents against various potato diseases, such as *B. subtilis* or *P. fluorescens* (Leben *et al.*, 1987; Sharga & Lyon, 1998). *Lysobacter enzymogenes* was reported as an antagonist of both *Pythium aphanidermatum* and *R. solani* (Folman *et al.*, 2003; Kilic-Ekici & Yuen, 2003), whereas *E. adhaerens* was only described as an endophyte of potato plants by Garbeva *et al.* (2001). Among the isolates with strong *in vitro* antagonistic activity, some belonged to putative potato pathogens such as *D. chrysanthemi*, causing tuber soft rot (Pérombelon, 1992), or *Streptomyces acidiscabies*, causing potato scab disease (Lambert & Loria, 1989). Most of the strains in this study antagonized *P. infestans*, a pathogen that is mainly distributed in temperate regions by latent infection of seed tubers (Johnson & Cummings, 2009). In an early study, uncharacterized bacteria on the tuber surface were found to antagonize *P. infestans* (Clulow *et al.*, 1994).

The cultivation-independent PCR-DGGE fingerprints revealed an effect of the plant genotype on the *Pseudomonas* community. Despite the significance of this effect ($P < 0.001$), the fingerprints differed only by 3.4%. In addition, the *Pseudomonas* patterns were almost identical between the plant genotypes at the Oberviehhausen site. Previously, it was observed that the separation of *gacA* genes in DGGE fingerprints has a superior taxonomic resolution compared with the 16S rRNA gene of the genus *Pseudomonas* (Costa *et al.*, 2007). However, in our study, the *gacA* fingerprints were more similar than the ribosomal fingerprints. This suggests that differentiating ribotypes were not included or not separated in *gacA* fingerprints. Overall, the high similarities of the fingerprints from the various taxa suggested a fairly subtle influence of the plant genotype on the tuber-associated bacteria. In contrast, environmental factors differing between both field sites had a much more pronounced effect on these bacterial communities.

Bands in the community profiles were found that comigrated with PCR fragments of major antagonistic strains of the genera *Bacillus* and *Streptomyces*. This may suggest that these antagonists were among the dominant members of the respective community. Indeed, the correlation of band intensity and isolation frequency in the case of *B. subtilis* 591 supported this assumption. However, comigrating bands do not necessarily represent the same species. It has also to be considered that strains of the same ribotype might differ in their antagonistic potential.

In conclusion, we found some evidence that the plant genotype influences tuber-associated bacterial communities. Noticeable observations were (1) the significantly lower numbers of culturable bacteria on tubers of the cultivar 'Selma' in the Roggenstein field experiment; (2) the increased numbers of strains with antagonistic potential for the cultivar 'Ditta' and the GM line SR48 in Oberviehhausen; and (3) the significant differences between plant genotypes in the composition of the *Pseudomonas* community in Roggenstein mainly with respect to the cultivar 'Sibu', as revealed by DGGE fingerprinting. However, these observed effects were not consistently related to a particular cultivar. In addition, the same effects were not found for both field experiments, probably because the genotype effect is strongly influenced by vastly differing environmental factors such as soil type or climate. The results suggest a rather weak effect of the investigated plant genotypes on the tuber-associated bacterial communities, and a clear consistent effect of the genetic modification of the zeaxanthin-accumulating plants was not observed. Effects of the plant genotype might be better detectable if potato varieties with known tolerance to phytopathogens were included in the survey, and if more targeted methods for differentiating phytopathogenic and antagonistic populations were applied.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Molecular characterization and 16S rRNA gene sequence analysis of antagonistic isolates from the tuber surface.

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